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Viewpoint Editorial

ERα, ERβ, and gpER: novel aspects of oestrogen receptor signalling in atherosclerosis

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Cardiovascular disease represents the leading cause of morbidity and mortality in women. Epidemiological and experimental evidence indicates several atheroprotective effects of endogenous oestrogens, which interfere with atherosclerosis progression and inflammation. Nevertheless, oestrogen receptor function across different stages of atherosclerosis development remains poorly characterized. This viewpoint editorial discusses the current knowledge of oestrogen receptor function in physiology and within different stages of atherogenesis. Increasing our understanding of the molecular mechanisms determining oestrogen action in humans, with and without established atherosclerosis, may help to develop new strategies for the treatment of cardiovascular disease in women, and possibly also in men.

1. Endogenous oestrogens and cardiovascular risk: role of menopause

Despite substantial efforts to improve education and public awareness and despite the use of effective medications and life-style changes for controlling the associated risk factors—hypertension, tobacco use, hypercholesterolemia, obesity, and diabetes—cardiovascular disease remains the leading cause of death in women worldwide.[1,2] In contrast to age-matched men, the incidence of clinical manifestations of coronary artery disease is considerably lower in premenopausal women; indeed, about 95% of women develop cardiovascular disease after menopause when endogenous oestrogen levels are low.[3–5] Accordingly, endogenous oestrogen protects from accelerated atherogenesis due to obesity in female teenagers and young adults compared with their male counterparts.[6] Cardiovascular risk increases after bilateral ovariectomy and in conditions associated with impaired ovarian function.[3,7] Thus, ovarian dysfunction and either natural or surgical menopause have been recognized as a major risk factor for accelerated atherosclerotic vascular disease development. In addition, cardiovascular risk factors and certain polymorphisms in oestrogen receptor genes may also determine an earlier onset of menopause.[8] Given that the number of postmenopausal women is expected to rise by 1 billion worldwide within the next 40 years,[9] understanding the impact of menopause on women's cardiovascular health has become increasingly important.

In addition to the epidemiological evidence, numerous *in vitro* studies have identified mechanisms whereby oestrogens exert beneficial effects on the cardiovascular system.[10,11] *In vivo*, the presence of endogenous oestrogens and their effect on cardiovascular homeostasis appear to be closely related to the degree of atherosclerosis progression throughout a woman's life.[12] Reversible fatty streak lesions are present *in utero*, [13] and 13% of US women aged 30–34 years already display advanced coronary artery lesions susceptible to rupture.[14] In stages of disrupted ovulatory cycling, low levels of endogenous oestrogens during premenopausal years accelerate the progression of atherosclerosis,[12,15] which can be reversed by oestrogen therapy in animals.[12] In addition, results from experimental studies and recent clinical trials indicate that oestrogen therapy started within few years after menopause, i.e. before the development of severe atherosclerosis, may in fact reduce cardiovascular risk.[5,16–20] In contrast, initiation of oestrogen therapy many years after menopause, i.e. when advanced and multiple atherosclerotic lesions are present, may have no or even deleterious cardiovascular effects.[5,16–20] Thus, the vascular response to endogenous and exogenous oestrogens appears to change with ageing and the presence or progression of atherosclerosis: while oestrogen could prevent the development of atherosclerotic plaques in newly menopausal women, it would have no or even harmful effects in older postmenopausal women with advanced atherosclerotic disease and vulnerable plaques. This has been referred to as the 'timing hypothesis'.[5,16–20]

2. Vascular oestrogen receptor signalling in women and men

Biological effects of oestrogens are mediated by three oestrogen receptors: oestrogen receptor-α (ERα), oestrogen receptor-β (ERβ), and the intracellular, transmembrane G protein-coupled oestrogen receptor (gpER).[21] The 'classical' ERα and ERβ receptors act as ligand-activated transcription factors that reside in the cytosol and translocate into the nucleus upon ligand binding. Subsequently, oestrogen

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receptors interact with oestrogen response elements in the promoter region of target genes.[11] In addition, oestrogens bind to plasma membrane-associated subpopulations of ERα and ERβ, thereby activating a variety of rapid intracellular signalling cascades.[21,22]

Oestrogens also activate gpER, previously termed GPR30, which is located to the endoplasmic reticulum and mediates rapid oestrogen signalling.[23] gpER is widely expressed in human tissues, including the cardiovascular system.[24,25] Interestingly, oestrogen signalling in cardiomyocytes involves ERα- and ERβ-independent pathways,[26] and treatment with the oestrogen receptor antagonists ICI 182,780 and tamoxifen, which are agonists of gpER,[27] inhibits cardiac cell growth.[28] Although genetic linkage studies indicated a potential role of the gpER locus on human chromosome 7p22 in the susceptibility to low-renin hypertension,[29] the role of gpER for vascular homeostasis remained unclear. We recently found that selective gpER activation acutely dilates human arteries, and markedly reduces blood pressure even under normotensive conditions, indicating that this receptor indeed controls vascular tone.[30] Moreover, selective gpER activation potently inhibits human vascular smooth muscle cell growth,[30] (*Figure 1*) consistent with the growth-inhibitory effect of ICI 182,780 and tamoxifen in cardiomyocytes.[28] The clinical significance of gpER as a mediator of the vascular protective effects of oestrogens in humans remains to be determined;[21] however, a recent subanalysis of the Raloxifene Use for The Heart (RUTH) trial suggests that treatment with a gpER agonist (raloxifene) may reduce cardiovascular risk in younger postmenopausal women.[31]

ERα, ERβ, and gpER are expressed in the arterial wall of both women and men,[11,25] and the non-selective oestrogen receptor agonist 17β-estradiol has potent dilator effects on vascular tone of human coronary and internal mammary arteries obtained from either female or male patients.[25,32]

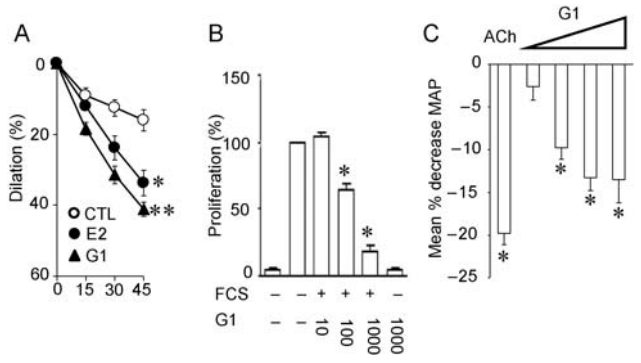

Figure 1 Involvement of gpER in regulation of vascular function, vascular smooth muscle cell growth, and blood pressure. The non-selective oestrogen receptor agonist 17β-estradiol (E2) and the selective gpER agonist G-1 acutely induce a relaxant response in human internal mammary arteries when compared with solvent control (CTL). However, the dilator effect of G-1 is even more potent than E2 (*A*). gpER activation also potently and concentration-dependently inhibits serum-stimulated cell proliferation in human vascular smooth muscle cells (*B*). Intravenous infusion of G-1 at increasing concentrations reduces mean arterial blood pressure (MAP) in normotensive Sprague–Dawley rats. For comparison, the pressure response to acetylcholine (ACh) is shown (*C*). Figure reproduced in part from Haas, E., Bhattacharya, I., Brailoiu, E., Damjanovic, M., Brailoiu, G.C, Gao, X., Mueller-Guerre, L., Marjon, N.A., Gut, A., Minotti, R., Meyer, M.R, Amann, K., Ammann, E., Perez-Dominguez, A., Genoni, M., Clegg, D.J., Dun, N.J., Resta, T.C., Prossnitz, E.R., Barton, M., 2009. Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. Circulation Research. 104, 288–291.[30] © 2009 American Heart Association.

These findings suggest a potential role for oestrogen receptor function also in the male cardiovascular system. Interestingly, 80% of plasma-bound 17β-estradiol in men originates from aromatization of testosterone and androstenedione in peripheral tissues by the enzyme aromatase,[33] which is highly expressed in the male vasculature.[34] Moreover, aromatase is expressed at high levels in the endoplasmic reticulum,[35] to which gpER is located.[23] Pharmacological inhibition of aromatase accelerates the formation of atherosclerotic lesions in male animals,[36] and impairs flow-mediated vasodilation in young men.[37] Moreover, male mice lacking functional ERβ develop hypertension.[38] In line with the finding that ERα mediates atheroprotective effects,[39] impaired vascular function and premature coronary artery disease were noted in a man with a disruptive mutation in the ERα gene.[40] Thus, not only the female but also the male cardiovascular system appears to be an important source and target for oestrogens affecting vascular disease development.[11,41] Nevertheless, studies in humans comparing oestrogen plasma concentrations and the progression of cardiovascular disease revealed conflicting results.[41] At present, there is doubt that treatment of male patients with oestrogen receptor-activating compounds, as shown experimentally in mice[42] and previously unsuccessfully attempted in male patients,[43,44] represents a treatment option to interfere with atherosclerosis progression. However, 17β-estradiol has been successfully used in male patients with coronary artery disease as a coating agent for endovascular stents, as indicated by the promising results of the Estrogen And Stents To Eliminate Restenosis (EASTER) trial.[45]

3. Does the development of atherosclerosis affect oestrogen receptor function?

Experimental studies suggest that at least in mice ERα appears to be largely responsible for the protective effects of oestrogens against atherosclerotic vascular disease.[39] On the other hand, expression of ERβ in humans correlates with coronary calcification and atherosclerosis independent of age.[46] Indeed, ERβ is expressed at higher levels than ERα in postmenopausal women with advanced atherosclerosis.[46] Moreover, ERβ expression is up-regulated following balloon angioplasty of structurally normal arteries of mice.[47] However, the absence of atherosclerosis makes the clinical relevance of this experimental model questionable. Interestingly, ERβ exhibits an inhibitory action on ERα-dependent gene expression and may oppose the actions of ERα, including its effects on facilitating nitric oxide-dependent, endothelium-mediated relaxation.[48,49] This could explain the loss of oestrogen responsiveness of arteries with atherosclerosis. Conversely, both ERβ deficiency in laboratory animals as well as functionally relevant mutations of the ERβ gene in women have been linked to the development of hypertension,[38,50] and associations of two ERβ polymorphisms with an increased cardiovascular risk in women have been reported.[51] It has also been recently demonstrated that ERα and ERβ regulate distinct and largely non-overlapping sets of genes in the arterial vascular wall.[52] Thus, both ERα and ERβ may have distinct roles ensuring vascular homeostasis that may, however, be affected by the presence of atherosclerosis.

According to some studies, the abundance of both oestrogen receptor subtypes ERα and ERβ in the aorta of humans decreases with the progression of atherosclerosis.[53,54] Inactivation of ERα and ERβ genes in the vasculature may be caused by DNA methylation of the promoter region of these genes; DNA methylation, an important epigenetic mechanism, increases with ageing and plaque progression.[55–57] Thus, structural changes of oestrogen receptors may result in functional loss of responsiveness to oestrogens. Indeed, proliferating vascular smooth muscle cells, which have undergone phenotypic modulation upon migrating to the intima,[58] display a high degree of ERα methylation.[56] However, we currently do not know whether and by which mechanisms changes in oestrogen receptor expression and structure in vascular cells affect atherogenesis, or whether vascular cells without functional oestrogen receptors become more abundant and prevent effects of oestrogens from becoming functional.

It has been suggested that the local formation of biologically active estrogens is higher in human aortas with mild atherosclerotic changes than in those with more advanced lesions.[59] Moreover, the ERβ-associated co-regulatory protein NM23-H2, which activates ERβ signalling *in vitro*, is down-regulated with progression of atherosclerosis in coronary arteries.[60] The notion that the protective effects of vascular oestrogen signalling are lost with the progression of atherosclerosis is further supported by the finding that 27-hydroxycholesterol, an abundant cholesterol metabolite found in atherosclerotic lesions, acts as an oestrogen receptor antagonist.[61] In addition, the aforementioned inactivation of oestrogen receptor genes, an altered balance of ERα and ERβ protein expression, and changes in oestrogen receptor signalling during different steps of atherogenesis may add up to a different regulation of oestrogen-sensitive genes, as well as of vascular homeostasis in general (*Figure 2*). Oestrogen therapy may also have beneficial effects on the expression and function of genes regulating calcium homeostasis that are limited to early, but not advanced, stages of atherosclerotic lesions.[19] Moreover, oestrogen-dependent induction of matrix metalloproteinases, proteolytic enzymes capable of degrading components of the extracellular matrix expressed in fibrous caps, may contribute to maintenance of lumen size in early atherosclerosis, but could increase the likelihood of plaque erosion or rupture in more advanced stages of the disease.[19,62]

Animal studies by Oparil's group have found that—unlike in young ovariectomized rats—oestrogen therapy in aged animals fails to prevent neointima formation and inflammatory responses following balloon angioplasty in structurally normal arteries.[63] This indicates that ageing—even in the absence of atherosclerosis—appears to modulate the chronic vascular response to natural oestrogens.[18] It has also been suggested that oestrogens, under certain conditions such as prolonged oestrogen deficiency, and particularly when used in the context of vascular inflammation as seen in atherosclerosis, may attenuate vasodilator capacity, enhance inflammatory activity, and improve plaque instability.[64,65] Accordingly, a prolonged period of hypoestrogenicity disrupts both neuroprotective and anti-inflammatory actions of oestrogens in animals.[66] In addition, oestrogen-dependent direct and endothelium-mediated relaxation as well as up-regulation of ERα and eNOS protein levels in ovariectomized rats is maintained when 17β-estradiol treatment is initiated 4 months, but not 8 months after surgery.[67] Together, these experimental studies provide evidence to support the concept of the 'timing hypothesis'.

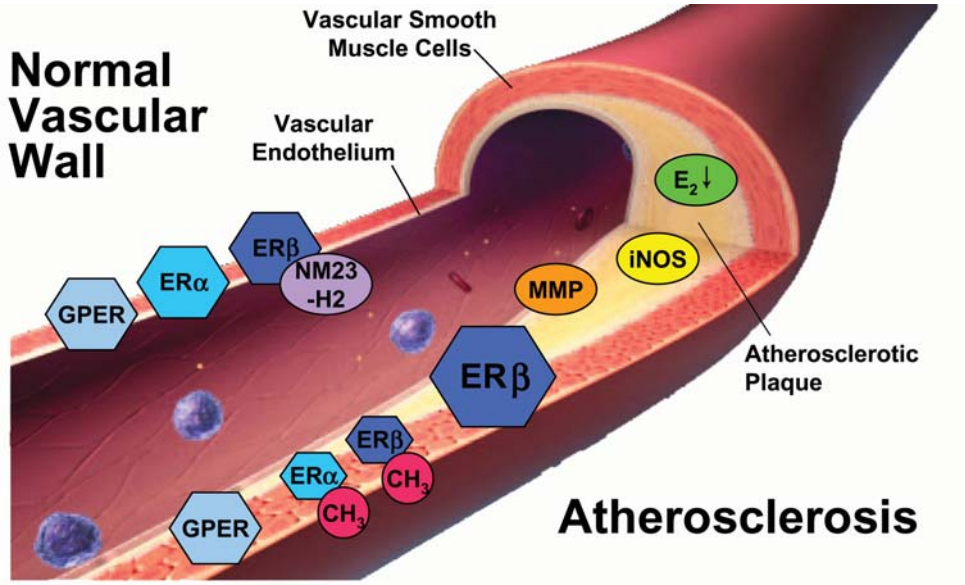

Figure 2 Molecular targets of oestrogens in the normal (left) and atherosclerotic vascular wall (right). With the progression of an atherosclerotic lesion as well as with ageing, expression of ERα and ERβ becomes a target of methylation (CH_3)-associated inactivation. Moreover, expression of oestrogen receptor-associated co-regulatory proteins, such as NM23-H2, is altered, and ERβ expression changes in advanced atherosclerosis. Local formation of biologically active oestrogens through aromatase and other pathways appears to be reduced ($E_2 \downarrow$) under these conditions. Based on these changes of oestrogen receptor signalling in advanced atherosclerosis, it is currently not clear how endogenous oestrogens affect oestrogen-dependent proteins involved in inflammation and structure of the atherosclerotic plaque, such as inflammatory nitric oxide synthase (iNOS) and matrix metalloproteinases (MMP). This illustrates the complexity of action of oestrogens and how equine oestrogen mixtures containing hormonal substances of unidentified activity affect oestrogen receptor function and regulation of oestrogen-sensitive genes in the presence of atherosclerosis. gpER, G protein-coupled estrogen receptor.

4. Clinical implications and perspectives

Although there is now substantial evidence suggesting that vascular functionality of oestrogen receptors depends on the length of a period of hypoestrogenicity and the degree of atherosclerosis progression, it is still not known how the cessation of endogenous oestrogen production after menopause affects oestrogen receptor signalling in human vascular cells *in vivo*. Therefore, future basic research should include studies that will help identifying the nature of changes of oestrogen receptor function in diseased arteries. Such changes were not considered in earlier randomized hormone therapy trials such as the Heart and Estrogen/progestin Replacement Study (HERS) and the Women's Health Initiative (WHI), which demonstrated adverse cardiovascular outcomes of hormone therapy in women late after menopause.[68,69] The medical regimen used in these studies consisted of conjugated equine oestrogens derived from horse urine, and of synthetic progestins such as medroxyprogesterone acetate.[68,69] Unlike 17β-estradiol, the major endogenous human oestrogen lost after menopause, conjugated equine estrogens contain a mixture of at least 10 different oestrogens, several androgens, progestins, and other substances of yet unknown vascular and procoagulatory activity.[5,18,41] These substances display different or even unknown selectivity and binding affinity for oestrogen receptors,[5] which result in unpredictable effects on multiple oestrogen signalling pathways evoking random vascular responses *in vivo*, especially in the presence of atherosclerosis. Moreover, medroxyprogesterone acetate has been reported to abrogate beneficial effects of oestrogens on vascular smooth muscle cell growth, lipid profile, and vascular function, even turning oestrogen-induced vasodilation into coronary vasospasm.[70–72]

Currently, prospective clinical trials are underway to test whether natural oestrogen has therapeutic potential in women with atherosclerosis: The Kronos Early Estrogen Protection Study (KEEPS; ClinicalTrials.gov number, NCT00154180), and the Early vs. Late Intervention Trial With Estradiol (ELITE; ClinicalTrials.gov number, NCT00114517). Moreover, the use of natural oestrogens such as 17β-estradiol at low doses, and possibly intermittent administration mimicking the normal menstrual cycle,[18,41] potentially will help to preserve activating signalling cascades, which are responsible for the beneficial vascular effects of oestrogens in premenopausal women. Finally, the use of selective oestrogen receptor modulators such as raloxifene, which act as gpER agonists,[27] might be suitable for treatment and prevention of atherosclerotic vascular disease. Results from the RUTH trial support this notion, at least in younger postmenopausal women.[31]

At present, hormone therapy is not an option for primary or secondary prevention of cardiovascular disease in women. There are proven therapies such as simple changes in lifestyle including maintaining a normal body weight, a diet rich in fruits and vegetables, and regular physical exercise. These measures—although still underused—should be initiated as early as possible to reduce the burden of atherosclerotic vascular disease later in life.[2,18]

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